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Estimation of podocyte number: A comparison of methods

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Estimation of podocyte number: A comparison of methods.

Background. The podocyte is the focus of much research into the mechanisms of renal disease progression, and the number of podocytes per glomerulus has thus become a parameter of much interest. When counting podocytes, the actual particle counted is the cell nucleus. The majority of published studies estimating podocyte number have used the method of Weibel and Gomez (1962). This makes assumptions about the shape and size of the cell nuclei and therefore has an inherent bias. In our studies we have used a more recent stereologic method—the disector/fractionator—that makes no assumptions about the shape or size of the cell nuclei and is therefore free of bias.

Methods. We set out to compare the two methods, in both type 1 diabetic patients and normal controls, to determine whether eliminating bias and thus improving accuracy had any effect on the overall results. The Weibel-Gomez method estimates cell number from a single section through the glomerulus, whereas the disector/fractionator requires the glomerulus to be serially sectioned.

Results. There was no significant difference between mean values obtained by the two methods, providing that the Weibel-Gomez estimate was performed on electron micrographs. However, the overall variance was high for all groups of patients, independent of the method employed.

Conclusion. Although the disector/fractionator is the theoretic gold standard method for podocyte number estimation, comparable estimates can be obtained by the Weibel-Gomez method provided they are made from electron micrographs. Thus the technical resources available may determine the choice of method employed. Investigators should be aware of the high degree of variability in the estimate, particularly when trying to detect small changes in podocyte number.

Recently, the podocyte has become the focus of much research into the mechanisms of progressive nephropathies. It has been proposed that damage to this cell is instrumental in the progression of such kidney diseases as focal segmental glomerulosclerosis (FSGS) [1], diabetic nephropathy [2, 3], and IgA nephropathy [4].

Key words: Weibel-Gomez, disector, fractionator.

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A proposed hypothesis is that podocyte loss results in initial widening of remnant foot processes and denuded areas of glomerular basement membrane (GBM). Both of these adaptations would lead to leakiness of the filtration barrier and increased proteinuria. Early supportive evidence for this hypothesis came from ultrastructural examination of the glomerular tuft revealing foot process widening, denuded GBM and tuft adhesion in various pathologic conditions [5]. Further evidence came from studies that examined urine sediments using immunocytochemistry [6]. These detected substances related to podocytes—podocalyxin, α_3-integrin, and C3b receptor—in the sediment of patients with a range of glomerular diseases. The same group has also quantified the number of urinary podocytes found in samples from patients with FSGS using the anti-podocalyxin antibody [7]. They found an average of 0.2 podocytes/mL in diseased patients, with one patient excreting 40 podocytes/mL.

In recent years, there have been several papers that have estimated the average number of podocytes in glomeruli from biopsy material [2–4, 8–10]. However, these studies have used different methodologies and the resulting numbers have not been consistent. As this is an area of increasing interest, we believe that it is important that there is a broad understanding of the relative strengths and weaknesses of the various methods that have been used.

METHODS

Estimation of number

Number density. The method of number estimation most used requires estimation of the numerical density (N_V) of the particles of interest and then multiplication of this by the reference volume (V) in order to derive an absolute quantity. This is often written as:

$$N = N_V \times V \qquad \text{(equation 1)}$$

However, there is a potential bias in this method. When counting particles it is necessary to give each of them an equal chance of being sampled. Particles in three-dimensional space that are cut by a two-dimensional section will be seen as profiles. Each section will hit a particle

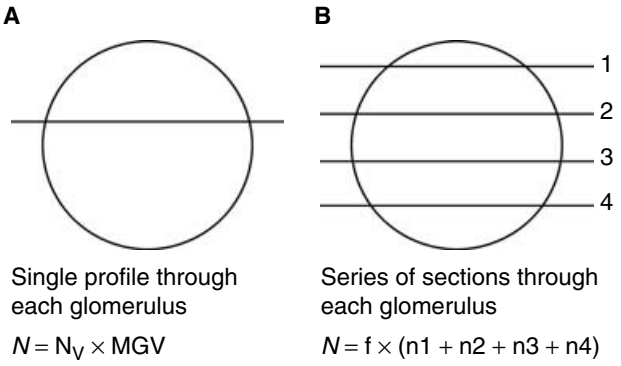

Fig. 1. Illustration demonstrating the sectioning of glomeruli for the estimation of podocyte number using (*A*) Weibel-Gomez and (*B*) fractionator method. Abbreviations are: N, total number of cells per glomerulus; N_V, number density; MGV, mean glomerular volume; f, inverse of sampling fraction; n, number of cells per profile.

in proportion to its size and, more specifically its height normal to the section. This will therefore bias against smaller particles in a population.

The method of Weibel and Gomez [11, 12] has attempted to overcome some of these problems using the equation:

$$N_V = K/\beta \sqrt{N_A^3/V_v} \qquad \text{(equation 2)}$$

where N_A is the profile density of the particles, Vv is the volume fraction of the particles, K is a size distribution coefficient, and β is a shape constant. For most biologic applications the size distribution coefficient, K, varies between 1 and 1.05 and can therefore be neglected [12].

Measurements are performed on a single section through the glomerulus (Fig. 1A). A grid of coarse and fine points (ratio 1:X) is superimposed onto the glomerular profile. The numbers of podocyte nuclear profiles (*N*) (assuming one nucleus per cell) and coarse points (P_{tuft}) hitting the glomerular tuft are counted in order to estimate podocyte nuclear profile density (N_A):

$$N_A = \Sigma n/[\Sigma P_{tuft} \times \text{ area per point}] \qquad \text{(equation 3)}$$

The number of fine points (P_{cell}) hitting podocyte nuclear profiles are counted to calculate the volume fraction of podocyte nuclei in glomerular tuft (V_V):

$$V_V = \Sigma P_{cell}/(\Sigma P_{tuft} \times X) \qquad \text{(equation 4)}$$

where X is the ratio of coarse to fine points.

The number density of cells can then be calculated from equation 2, with a shape constant chosen depending upon whether the cells are considered to be spherical, an ellipse, or an ellipsoid. The shape constant used in this analysis was for an ellipsoid, 1.55.

This method therefore makes a priori assumptions about shape and therefore cannot be regarded as being truly unbiased. Podocyte nuclei have a very complex shape that may change with disease, which the Weibel-Gomez method cannot accommodate.

Table 1. Comparison of average podocyte number per glomerulus estimated in control patients from different studies

	Number	Podocyte number	CV	CE
Pagtalunan et al [2]	8	575 ± 127	0.22	0.08
Steffes et al [8]	24	878 ± 220	0.25	0.05
Lemley et al [4]	14	300 ± 107[a]	0.36	0.10
White et al [9]	10	580 ± 129	0.22	0.07
Dalla Vestra et al [10]	20	833 ± 184	0.22	0.05

CV, coefficient of variation; CE, coefficient of error.
Data are mean ± SD.
[a]Paraffin-embedded tissue.

In addition, once the estimation of N_V is obtained, there is still a potential bias depending upon the method chosen for estimation of glomerular volume. Several methods have been used, with varying levels of precision.

Lane, Steffes, and Mauer [13] compared four methods for estimating glomerular volume—Cavalieri [14], maximal profile area (MPA), Weibel and Gomez [11, 12], and the disector principle [15]. The MPA and Weibel-Gomez methods assume the glomerulus is either a sphere [13] or an ellipsoid [16]. Therefore, although they attempt to correct for this assumption, a further bias is introduced. The Cavalieri method is considered to be the gold standard method for estimating volume, as it makes no assumptions about the size or shape of the glomerulus. The disector method is also free of bias but is generally more useful for the estimation of number rather than volume.

Any bias introduced by the method chosen for estimation of number density and volume may be amplified depending upon the tissue processing method. For example, embedding in paraffin results in greater overall tissue shrinkage than resin. Thus, if estimates of glomerular volume are obtained from paraffin-embedded tissue but podocyte number density is estimated in resin-embedded tissue, it follows that when multiplying number density by glomerular volume a smaller absolute number will result than if both measurements are obtained from resin-embedded tissue. Although this may not affect the results of an individual study where like is compared with like, it makes comparisons between studies more difficult. This is demonstrated in Table 1, where published data from control subjects show a wide variation. The average number of podocytes per glomerulus ranges from 300 ± 107 [4] to 878 ± 220 [8]. The tissue used for glomerular volume estimation in the study of IgA nephropathy [4] was paraffin-embedded (podocyte number = 300 ± 107), while the other studies used resin-embedded tissue (podocyte number = 575 ± 127 to 878 ± 220).

Disector/fractionator. An alternative unbiased method for the estimation of number is the disector/

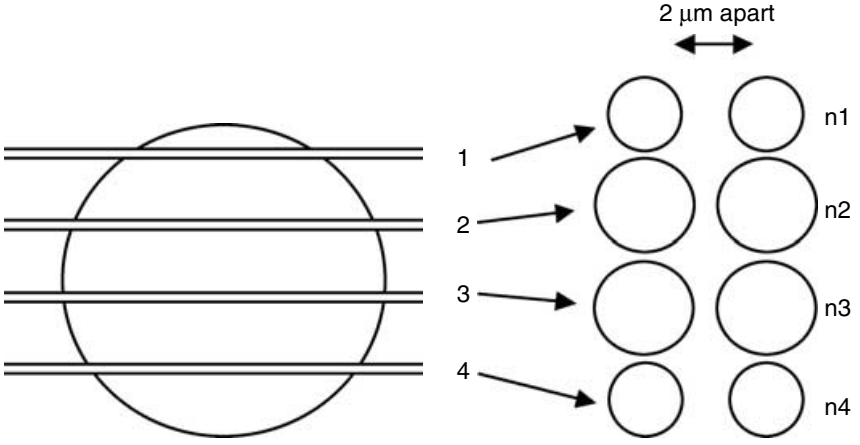

Fig. 2. Illustration demonstrating the sectioning of glomeruli for the estimation of podocyte number using the disector/fractionator combination. Abbreviations are: N, total number of cells per glomerulus; Q^-, number of cells counted; f, inverse of sampling fraction = 10 × 6; n, number of cells per profile; t, distance between profile pairs = 2 μm. Q^- is divided by 2 as the disector is counted in both directions.

fractionator combination [9, 17], which is independent of both the size and shape of the particle and its reference volume.

The basic principle of the fractionator is quite simple. If the number of particles in a known fraction of the whole object is counted, multiplying that number by the sampling fraction will give an unbiased estimate of the total number (Fig. 1B). For example, in the case of podocyte number, 1 μm serial sections are taken through the glomerulus and every *tenth* section collected, resulting in approximately 20 profiles per glomerulus. Then, starting at a random point, every *sixth* profile is selected, resulting in three to four profiles per glomerulus with a distance between levels of 60 μm. Podocyte nuclear profile number is counted on these profiles and then multiplied by the inverse of the sampling fractions to give an estimate of the total number of cells per glomerulus (i.e., total number = number counted × 10 × 6).

Podocyte nuclei are counted using the disector principle [15]. The only variable that needs to be known is the distance between adjacent disector pairs (Fig. 2).

A protocol for the estimation of podocyte cell number per glomerulus is as follows. Starting at a random sectioning level within the glomerulus, two adjacent glomerular profiles in serial sections are selected at a set interval, or fraction, through the glomerulus and each pair viewed side by side on a computer screen. Those podocyte nuclei appearing in one profile (the reference section) but not the other (look-up section) are counted. The efficiency of the disector can be doubled by then swapping the roles played by the reference and look-up sections. It is impossible for a nucleus counted in one direction to also be counted in the opposite direction.

The number of cells can then be estimated:

$$N = (f_1 \times f_2 \times \ldots . f_k)/t \times Q^-/2 \quad \text{(equation 5)}$$

where f_k is the sampling period (reciprocal of the sampling fraction) at the lowest sampling stage, t is the distance between adjacent profiles, and Q^- is the total number of cell nuclei counted (divided by 2 as the disector is counted in both directions).

The distance between the adjacent profiles must be smaller than the diameter of the nuclei measured, but not so small that there is no discernable difference between the two sections. The distance selected in our laboratory is 2 μm. For maximum efficiency, the average Q^- per biopsy should be approximately 100.

The accuracy of a microtome for cutting 1 μm sections can be assessed by cutting a 10 μm "terrace" in the side of a resin block in 1 μm steps, then measuring the distance between the block face and terrace by cutting 90 nm gold ultrathin sections. It should take approximately 111 ultrathin sections to reach the terrace.

We directly compared the Weibel-Gomez and disector/fractionator methods by estimating podocyte number in 16 type 1 diabetic patients at baseline and after 3 years, and 10 normal controls. Measurements were carried out on five glomeruli per biopsy. Estimations were performed in the same glomeruli for each method. For light microscopy the magnification used was ×1370 and for electron microscopy ×2000. For the Weibel-Gomez method, glomerular volume was estimated using the Cavalieri principle. All tissue was fixed in glutaraldehyde, post-fixed in osmium tetraoxide, and embedded in epoxy resin.

The disector/fractionator method was performed using light microscopy and the Weibel-Gomez method on both light and electron microscopy. The disector/fractionator technique was also performed using electron microscopy on a few glomeruli. However, it proved very difficult to ensure that the distance between adjacent profile pairs was 2 μm and therefore this method was not considered

Table 2. Podocyte number per glomerulus estimated using both the disector/fractionator and Weibel-Gomez methods in 16 type 1 diabetic patients and 10 control subjects

		Disector/fractionator (light)		Weibel-Gomez (light)		Weibel-Gomez (electron)	
	n	Mean (95% CI)	CV	Mean (95% CI)	CV	Mean (95% CI)	CV
Type 1 patients baseline	16	544 (469–619)	0.26	339 (290–389)	0.27	524 (456–592)	0.24
Type 1 patients follow-up	16	475[a] (422–527)	0.21	355 (301–410)	0.29	467[a] (388–547)	0.32
Controls	10	580 (487–672)	0.22	383 (341–424)	0.15	616 (507–726)	0.25

CV, coefficient of variation.
[a] $P < 0.05$ versus corresponding control.

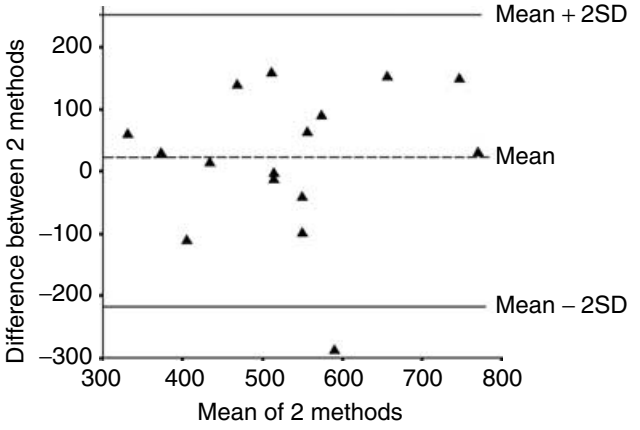

Fig. 3. Bland-Altman plot of difference against mean for podocyte number in 16 type 1 diabetic patients at baseline measured by disector/fractionator and Weibel-Gomez (electron microscopy).

to be a practical option for the estimation of cell number and was not included in further analysis.

RESULTS

It can be seen from the results that the disector/fractionator and Weibel-Gomez methods give similar values for podocyte number providing that the measurements for Weibel-Gomez are carried out using electron microscopy (Table 2). The values obtained using the Weibel-Gomez method on light microscopy sections are significantly lower ($P < 0.001$).

There are no significant differences between control subjects and diabetic patients at baseline using any of the three methods. At follow-up, however, both the disector/fractionator and Weibel-Gomez electron microscopy method show a significant reduction in podocyte number in diabetic patients compared to controls ($P = 0.028$, $P = 0.022$, respectively). There is no significant difference detected using the Weibel-Gomez light microscopy method ($P = 0.45$).

A Bland and Altman [18] plot shows that despite there being no statistically significant differences in podocyte number estimated by the Weibel-Gomez electron microscopy or disector/fractionator methods, there is, however, a lack of agreement of the estimate obtained by the two methods (Fig. 3). The mean difference in the type 1 patients at baseline is 20, with 95% CI of −43 to 83. In control patients there is no significant difference in the within-patient variability obtained by Weibel-Gomez electron microscopy or disector/fractionator. In the type 1 patients at baseline, however, the within-patient variability is significantly lower using the disector/fractionator ($P = 0.008$). This difference is not evident as disease progresses ($P = 0.113$).

DISCUSSION

Despite the inherent bias in the Weibel-Gomez method due to assumptions about the shape and size of podocyte nuclei, it would appear that a reasonable estimate of podocyte number may be obtained comparable to that observed with the unbiased disector/fractionator method—providing that electron micrographs are used for the Weibel estimation. The main drawback of the disector/fractionator is that it is much more time-consuming than the Weibel-Gomez method as it requires exhaustive serial sectioning of the glomerulus. It takes a minimum of 3 days to serially section and count five glomeruli per biopsy. However, although a single section through each glomerulus may take less time for the Weibel-Gomez method, obtaining electron micrographs of glomerular profiles is more expensive and obviously requires access to an electron microscope, something that is not available to all researchers. In addition, the Weibel-Gomez method may also require serial sectioning depending upon the method chosen for estimation of glomerular volume. In this case, the Weibel-Gomez method becomes less cost effective than the disector/fractionator. However, it should be noted that if electron microscopy is being used to estimate other glomerular parameters, the additional cost of estimating podocyte number is negligible.

The estimation of podocyte number using Weibel-Gomez and light microscopy appears to be further biased, although the reasons for this are not obvious. As the Weibel-Gomez method is performed on a single level through the glomerulus, the number of nuclear profiles counted can be quite low. Therefore, just a few missed

nuclei at the light compared to electron microscopy level may result in a proportionally greater difference in overall podocyte number. It may be possible to reduce this discrepancy by performing counts on multiple levels through the glomerulus; this, however, will also decrease the cost effectiveness of this method.

Although there is some evidence from looking at within-patient variability that the disector/fractionator method improves precision in diabetes, the overall high variance of the estimate may mean that these small increases in precision are of little practical consequence. The high variability of the estimate also suggests that small changes in podocyte number will be difficult to detect. Our recently published data show no significant difference in podocyte number in 50 type 1 diabetic patients at baseline compared to 10 controls, whereas there was in fact a 10% difference in absolute podocyte number [9]. After 3 years of follow-up a significant difference was detected, with an 18% reduction in absolute podocyte number.

CONCLUSION

The Weibel-Gomez method requires assumptions to be made regarding the shape and size of podocyte cell nuclei and therefore could be dismissed on the basis that they introduce unquantifiable bias in the estimate. However, in practical terms the actual estimates of podocyte number using the two methods are not statistically different; therefore, the method chosen by researchers may depend upon the available resources. The disector/fractionator offers the theoretic advantage of lack of bias and could therefore be considered as a gold standard. In addition, depending upon the method chosen for estimation of glomerular volume, it may be more cost effective. Investigators should be aware of the high variability of the estimate when planning studies looking at podocyte loss in pathologic disease processes.

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REFERENCES

1. Kriz W, Gretz N, Lemley KV: Progression of glomerular diseases: Is the podocyte the culprit? *Kidney Int* 54:687–697, 1998
2. Pagtalunan ME, Miller PL, Jumping-Eagle S, *et al*: Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99:342–348, 1997
3. Meyer TW, Bennett PH, Nelson RG: Podocyte number predicts long-term urinary albumin excretion in Pima Indians with type II diabetes and microalbuminuria. *Diabetologia* 42:1341–1344, 1999
4. Lemley KV, Lafayette RA, Safai M, *et al*: Podocytopenia and disease severity in IgA nephropathy. *Kidney Int* 61:1475–1485, 2002
5. Andrews P: Morphological alterations of the glomerular (visceral) epithelium in response to pathological and experimental situations. *J Electron Microsc Tech* 9:115–144, 1988
6. Hara M, Yanagihara T, Itoh M, *et al*: Immunohistochemical and urinary markers of podocyte injury. *Pediatr Nephrol* 12:43–48, 1998
7. Hara M, Yanagihara T, Kihara I: Urinary podocytes in primary focal segmental glomerulosclerosis. *Pediatr Nephrol* 89:342–347, 2001
8. Steffes MW, Schmidt D, McCrery R, Basgen JM: Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* 59:2104–2113, 2001
9. White KE, Bilous RW, Marshall SM, *et al*: Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* 51:3083–3089, 2002
10. Dalla Vestra M, Masiero A, Roiter AM, *et al*: Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 52:1031–1035, 2003
11. Weibel ER, Gomez DM: A principle for counting tissue structures on random sections. *J Appl Physiol* 17:343–348, 1962
12. Weibel ER: *Stereological Methods for Biological Morphometry* (vol 1), London, Academic Press, 1979, pp 44–45
13. Lane PH, Steffes MW, Mauer SM: Estimation of glomerular volume: A comparison of four methods. *Kidney Int* 41:1085–1089, 1992
14. Gundersen HJG, Bendtsen TF, Korbo L, *et al*: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 96:379–394, 1988
15. Sterio DC: The unbiased estimation of number and sizes of arbitrary particle using the disector. *J Microsc* 134:127–136, 1984
16. Pagtalunan ME, Drachman JA, Meyer TW: Methods for estimating the volume of individual glomeruli. *Kidney Int* 57:2644–2649, 2000
17. Nyengaard JR: Stereologic methods and their application in kidney research. *J Am Soc Nephrol* 10:1100–1123, 1999
18. Bland JM, Altman DG: Statistical methods for assessing agreement between methods of clinical measurement. *Lancet* 1:307–310, 1986